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When the Mediterranean becomes harsh: Heat pulses strongly affect C allocation in plant-soil-atmosphere continuum in *Eucalyptus camaldulensis*

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ABSTRACT

Mediterranean vegetation is frequently subjected to the heat waves with temperature peaks exceeding 45 °C. While the effects of drought on assimilates allocation are deeply studied, little information is available regarding the effects of the acute heat stress on this aspect of the plant physiology. We evaluated the effects of acute heat pulses on plant C internal allocation in *Eucalyptus camaldulensis* saplings and its transfer into the soil. Shoots of *Eucalyptus* were subjected to an increase of the air temperatures up to 45 °C. Allocation of C was inferred after pulse labelling of heated and non-heated shoots with $^{13}CO_2$. Heat pulse lowered the photosynthesis and almost tripled the C losses from shoots through respiration. Assimilated ^{13}C was preferentially allocated to leaf soluble sugar fractions rather than to starch. Heat pulse interrupted ^{13}C allocation to the trunk, roots and soil. ^{13}C allocation to new non labelled leaves was detected in some replicate plants, independently of treatment, suggesting that this process is sink-driven. Although, shoots subjected to the heat pulse modified allocation strategy, "saving" assimilated ^{13}C at a leaf level, the rate of ^{13}C involvement in shoot respiration was restricted by photosynthetic rates measured during the labelling. The results suggest that photosynthetic and non-photosynthetic organs may be constrained to rely on C reserves under elevated temperatures with further negative consequences for C balance in case the duration and frequency of the heat waves will increase as projected by climate change.

1. Introduction

Plants grown in the Mediterranean region are subjected for the extended part of the year to a strong water deficit and high temperatures. These two events are essentially linked in summer and intensification of the one enhances the severity of another (positive feedback) (Stéfanon et al., 2014). Mediterranean vegetation possesses various mechanisms to cope with summer drought by down-regulating the metabolic activity or developing tools to avoid the stress (Farooq et al., 2009; Gavrichkova et al., 2018). *Eucalyptus* spp., one of the most common genus in Mediterranean especially in forest plantations, tolerate extended drought periods as well. It is achieved by shedding a part of the canopy (mature leaves and twigs) in order to reduce transpiration rate and/or by developing deep root system with constant access to groundwater combined to considerable tolerance of salinity (Mensforth et al., 1994). Some warm events however, like the wind Sirocco in the Mediterranean, occur more frequently in spring and autumn when the water table is charged. Sirocco lasts from 0.5 to 7 days and carries the warm air from Sahara with peaks in air temperature reaching in some regions 43–46 °C (Goldreich, 2003; Galvin, 2015). Mediterranean vegetation in this period is at the peak of metabolic activity: actively growing in spring or recovering after the summer drought in autumn (Larcher, 2000; Peñuelas et al., 2002). These phenophases are more vulnerable and the consequences of the exposure to

Abbreviations: A, ambient; H, heated; A_{shoot}, shoot net CO_2 assimilation; A_{leaf}, leaf net CO_2 assimilation; R_{shoot}, shoot respiration; R_{trunk}, trunk respiration; R_{mic}, microbial respiration

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extreme temperatures could be long-lasting, affecting plant performance in the subsequent years (e.g. Heide, 2003; Yue et al., 2010). Climate change forecasts increase in the frequency, duration and severity of such extreme events globally and particularly in the Mediterranean region (Guirguis et al., 2014; Frank et al., 2015).

Physiology of heat stress have been studied on various levels of plant organization, from cellular to ecosystem (e.g. Gargallo-Garriga et al., 2015; Drake et al., 2018; Tatarinov et al., 2016). Leaf level survey on plants grown in various biomes reported wide margins of safety thermal tolerance for most plants with mid latitude vegetation being at highest risk of damage due to the narrowest thermal safety margins and most severe heat events (O'Sullivan et al., 2013). Experiences of the extreme years 2003 and 2010 suggest that strong heat waves in combination with prolonged drought are harmful for temperate and boreal vegetation mainly through indirect and lagged impacts. Being adapted to mild temperatures and sufficient water supply, the ecosystems in these regions lose C (Ciais et al., 2005; Bastos et al., 2013), become more susceptible to other stressors like insects' outbreak and forest fires resulting in considerable dieback of the vegetation (Allen et al., 2010).

Few ecosystem-scale evidences from Mediterranean vegetation suggest that during the heat waves the C-gain related parameters like net ecosystem exchange (NEE), gross primary production (GPP) and stomatal conductance (g_s) are decreasing, but plants are often highly resilient and recover fast without any long-term consequences (Tatarinov et al., 2016). The decline in NEE is mainly attributed to high respiration which boosts at elevated temperature and continues growing well beyond the temperature optimum for photosynthesis (Turnbull et al., 2002; O'Sullivan et al., 2013; Smith and Dukes, 2013; Yamori et al., 2014). Among mechanisms, which transmit thermal resistance to the plant could be named leaf latent cooling (Urban et al., 2017; Drake et al., 2018), reflective leaves (Curtis et al. 2012) and formation of heat shock proteins and BVOCs (Feder & Hofmann, 1999, Loreto and Schnitzler, 2010). Biomass is redistributed between organs in manipulative + 12 °C warming experiments with complex decline in plant biomass but increase in plant height of some species either decline of plant height and trunk diameter in the others (Bauweraerts et al., 2014). Activation of protection mechanisms, changes in plant C sequestration, growth dynamics and nutrient availability under heat waves points on variations in plant internal C allocation strategy. However, mechanistic understanding of C allocation changes in the conditions of the heat stress is lacking. This is also the case of the recent review by Teskey et al. (2015) which addressed the effects of the heat waves on trees functioning. Some analogues could be created with changes in C allocation in response to other stressors. Experiments with isotope tracers suggest that newly assimilated C is primary involved in ensuring plant metabolic activity in optimum growing conditions, while stored C is mobilized under severe stress (Carbone et al., 2013; Hasibeder et al., 2015; Muhr et al., 2016; Von Rein et al., 2016). Consumption of the reserves stored in plant compartments to cover the elevated respiration and thermal protection expenses could be hypothesized in the conditions of heat stress with consequences to whole plant and individual organs growth dynamic. Stress conditions, which inhibit sugars-consuming processes (ex. low temperature, drought, soil salinity), are often associated with accumulation of sugars and C reserves (Hartmann et al., 2013; Galiano Pérez et al., 2017; Gavrichkova et al., 2018). In case of severe heat, which instead stimulates respiration and sugar consumption, reserves and sugars accumulation should be instead compromised. Allocation of newly assimilated C to sink non-photosynthetic organs will depend on the photosynthetic rate of the source leaves itself (Barthel et al., 2011; White et al., 2015), but also on the capacity of the sink organs to attract C (Hoch and Keel, 2006; Mason et al., 2014; White et al., 2015) in the stress conditions.

We aimed to evaluate the effects of strong heat pulses on C allocation in *Eucalyptus camaldulensis*. Shoots of 4 yr-old saplings were subjected to an increase of the air temperature up to 45 °C during the day time. Changes in C allocation with the establishment of the heat was inferred after pulse labelling of heated and non-heated shoots in $^{13}CO_2$ enriched atmosphere and subsequent ^{13}C tracing in the main pools and fluxes in the plant-soil system. Label dynamic was assessed in shoot respiration, leaves, branches, trunk, soil, roots and soil microbial respiration.

The following hypotheses were tested:

1 Heat pulse will affect leaf gas exchange parameters differently with the drop of CO_2 assimilation and increase of respiration.
2 Leaf level C allocation: increase in leaf respiration flux will call for additional C, which will be mobilized from newly assimilated C and particularly from non labelled C reserves. The involvement of the reserves will be proportional to plant assimilation activity. At the same time, conversion of recent assimilates into C reserves will be compromised for the same reason.
3 Plant level C allocation: shoot heating will affect the distribution of recently assimilated C between organs and tissues, particularly ^{13}C will remain primary at the leaf and branch level to cover higher C requirements of shoot respiration. Contemporary, allocation to heterotrophic organs like trunk and to belowground pools like roots and soil will be compromised.

2. Materials & methods

2.1. Plants growing conditions, labelling and sampling

Ten 4-years old *Eucalyptus camaldulensis* saplings from the Eucalyptus germplasm collection of the research unit for intensive wood production of Council for Agricultural Research and Agricultural Economics Analysis (CREA-PLF) were transferred to Research Institute on Terrestrial Ecosystems of National Research Council (IRET-CNR) and grown for 6 months in 35 L pots in ventilated and open to the exterior environment glass house with regular water supply and natural photoperiod. Six healthy plants similar in structure and size (ca. 150 cm height) were selected and divided into two groups: the first – plants subjected to a heat pulse during the labelling (n = 3) and the second – labelled at ambient temperature (n = 3) and heated afterwards. Plants were labelled in June. To avoid time-associated effects, labelling on heated (hereafter as *H*) and non-heated ambient plants (hereafter as *A*) was carried out in rotation: ^{13}C labelling was performed each time on a single plant and lasted for 3 h with the subsequent chasing period of 54 h.

Labelling was performed in controlled laboratory conditions. Prior to the labelling, plants were kept for 24 h in laboratory for acclimation at 30/24 °C day/night temperature and photosynthetically active radiation (PPFD) at the top canopy of 1000 μmol m^{-2} s^{-2} in the day hours (Fig. 1). During this period one shoot located at the middle canopy height was placed in a labelling open-top transparent branch glass enclosure (1.7 L volume) equipped with light, humidity and temperature sensors and constantly flushed with 2 L min^{-1} moist gas mixture of N_2, O_2 and CO_2 at ambient temperature in proportion 80:20:0.04. The airflow was kept constant using Brooks 5850 series E mass flow controllers (Brooks Instrument, Veenendaal, Netherlands). The internal temperature of the enclosure was controlled by circulating water between the thermostatic bath and a chamber surrounding the enclosure (hereafter all the system as *branch chamber*). Relative humidity was controlled in the range of 50–60% by condensing the excess of water vapor in a glass coil immerged in a water bath. The VPD was constant during the experiment. Air temperature, relative humidity and PPFD were constantly monitored inside the branch chamber by the AM2315 sensor (Adafruit, New York, USA) and SQ110 (Apogee Instruments, Utah, USA), respectively. The desired PPFD flux was reached by regulating the distance between the lamp (Power Star lamp, OSRAM, Milano, Italy) and the branch chamber. All climatic data were acquired with 1 Hz frequency and logged in the datalogger CR6 (Campbell Scientific, UT, USA). See Pallozzi et al. (2016) for further information on

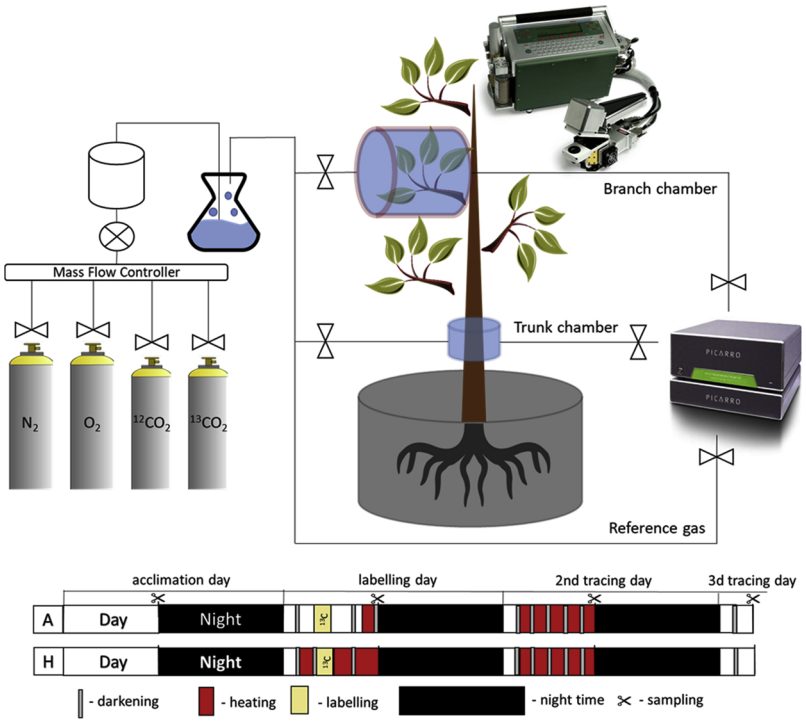

Fig. 1. Experimental set-up. Branch and trunk chambers were constantly flushed with the air mixture. Branch chamber was heated to 45 °C prior to ^{13}C pulse labelling in *H* treatment group. Plants in *A* group were labelled at ambient temperatures (30 °C). At the time of labelling, the gas tank with the CO_2 was replaced with the CO_2 at 99 at% ^{13}C. Labelling duration was 3 h with the subsequent chasing of the label in pools (for 54 h) and fluxes (for 48 h) along the C transfer path. Plants in *A* group were subjected to a short 45 °C heat pulse too, but only after the 3h-labelling event had been completed. Labelled shoot net CO_2 assimilation (A_{shoot}) and shoot respiration (R_{shoot}) as well as trunk respiration (R_{trunk}) were monitored with Picarro G2201 (Picarro Inc, Nebraska, USA). Leaf-level net CO_2 assimilation (A_{leaf}) and related parameters were monitored contemporary on non labelled shoots with Li6400 (Li-Cor Inc., NE, USA) at ambient (30 °C) and heated (45 °C) temperatures.

environmental condition controlling.

Another chamber (hereafter as *trunk chamber*, 1.5 L volume, TecnoEl, Rome, Italy, Fig. 1) was installed at the trunk level for trunk respiration measurements (R_{trunk}). The chamber was placed approximately 20 cm above the soil and flushed with the same gas mixture. Temperature and humidity in the chamber were constantly monitored with CS215 (Campbell Scientific, UT, USA).

Concentrations of the $^{12}CO_2$ and $^{13}CO_2$ isotopologues in the outlet of branch and trunk chambers were measured with Picarro G2201i analyzer (Picarro Inc, NE, USA, Fig. 1). 16-Port Distribution Manifold (Picarro Inc, NE, USA) was switching between the chambers and the gas mixture at the inlet with 6 min frequency and deploying the air to Picarro. The same gas mixture was used as an internal standard ($\delta^{13}C$ of -17.0‰) in order to monitor the system stability in isotope measurements. During the day time, at the end of each two complete cycles between all the chambers, the branch chamber was shaded for 20 min as suggested by Gratani et al. (2008) in order to measure shoot respiration in the dark (R_{shoot}).

After switching on the light in the labelling day, gas exchange parameters in the light (shoot net CO_2 assimilation, A_{shoot}) and in the dark (R_{shoot} and R_{trunk}) were first registered at ambient (30 °C) temperature. In order to simulate severe heat shock, the temperature in the branch chamber of *H* plants was raised and maintained during the day at approximately 45 °C by adjusting the water temperature in the thermostatic bath. The rest of the plant canopy was maintained at ambient temperature. Once the temperature was stabilized and gas exchange parameters in the light and in the dark reached apparent steady state at new conditions (approximately 1.5 h after heat on set), $^{12}CO_2$ in the mixture was replaced with 99% $^{13}CO_2$ (Sigma-Aldrich, Darmstadt, Germany). After 3 h of labelling, $^{13}CO_2$ was switched back to $^{12}CO_2$. Plants from *A* group were labelled in the same way but without changes in the air temperature. Post-labelling conditions were instead similar between the treatments (Fig. 1). After one complete cycle of measurements between the chambers at ambient temperatures, branches of *A* plants were heated up and kept at similar temperatures to *H* plants. Heat was kept on for *H* plants during the day hours and was turned in both treatments to ambient temperatures during the night. In the next day labelled shoots from both treatments were again heated up until night.

$\delta^{13}C$ of R_{trunk} and R_{shoot} was calculated using a two-end mixing model:

$$\delta^{13}C = \frac{(\delta Cout^{*}[Cout]) - (\delta Cin^{*}[Cin])}{([Cout] - [Cin])}$$

where $\delta Cout$ and δCin are $\delta^{13}C$ measured in the outlet and inlet of the respective chamber, and $[Cout]$ and $[Cin]$ are the CO_2 concentrations (sum of $^{13}CO_2$ and $^{12}CO_2$) in the same sample.

The ^{13}C excess (exC) in respiration flux at a certain time ($ex^{13}Ct$) was calculated as the difference between the ^{13}C atom % of the flux after labelling ($atom\%_s$) and its natural level measured before labelling ($atom\%_{bg}$). To obtain absolute values of exC, the atom % value was multiplied by the mass of the flux (C_{total}, mg m^{-2} h^{-1}):

$$ex^{13}C_t = \frac{atom\%_s - atom\%_{bg}}{100} * C_{total}$$

Leaves from shoots growing above, at the same height and below the labelled shoot were sampled 3 times, 6 h, 30 h and 54 h after the labelling, in order to limit the disturbance on the system (Fig. 1). Each subsequent sampling has never been performed on already sampled shoot. One of the most juvenile leaf was left intact during the sampling in order to maintain active the shoot sink capacity. Leaves were divided into old fully expanded and new not fully expanded leaves and frozen at -80 °C. At the same time, soil cores (2 cm in diameter and 20 cm deep) were sampled in two opposite sides of the pot. Created holes were filled with inert material. Soil was cleaned from roots, sieved to 2 mm mesh and maintained at −20 °C. Collected roots were kept at -80 °C prior to processing and analyses. Enrichment of microbial respiration (R_{mic}) was determined on 1 g of the sieved moist soil, which was placed in a 12 mL vial, flushed with the CO_2 free air for 5 min and incubated at a room temperature for 24 h. $\delta^{13}C$ of the respired CO_2 was analyzed on Multiflow coupled to IRMS Isoprime (Isoprime, Cheadle, UK). By the end of the chasing, labelled shoots were harvested, leaves were divided into new and old leaves and woody branches, and maintained as previously described.

Branch level of the labelling allows to stabilize the primary sinks for recently assimilated C and to evaluate the capacity of the leaves

subjected to the heat pulse to maintain their C-source function in respect to other organs, including growing leaves located on different canopy heights (hypothesis 3). Tracing of the label allocation within the canopy won't be possible in the case of whole-plant labelling. Heating of the control *A* shoots after the labelling was performed in order to increase the respiration expenses of leaves according to hypothesis 1. Because *A* plants are supposed to assimilate more label in respect to *H* plants (hypothesis 1), post-labelling heating allows to evaluate the potentiality of differently charged during the labelling branches (*A* versus *H*) to cover increased respiration expenses with labelled photosynthates (hypothesis 2). In the preliminary experiment performed on one *Eucalyptus* sapling tree at ambient conditions it was shown that the peak of $\delta^{13}C$ in R_{trunk} occurs in the first two days after the labelling (Fig. S1). Lag time, time to a maximum and maximum label in a pool are important parameters which are used to characterize the dynamic of recently assimilated C in this timeframe (Kuzyakov and Gavrichkova, 2010). These parameters are expected to be affected by the variation in shoot C allocation strategy during the labelling induced by the heat pulse. On the third day, enrichment of R_{trunk} exponentially declines (Fig. S1). This part of the curve determines the residence time of the label in a pool and is more dependent on internal to a pool processes (Kuzyakov and Gavrichkova, 2010). In our case the pool of interest, trunk, was not subjected to heating making this timeframe less interesting in current experimental set-up. By this reason it was decided to strengthen the attention on the first 48 h of the chasing period for respiration isofluxes. It permitted to increase the sampling frequency and to capture even small changes in diurnal variation of trunk and shoot isofluxes.

In order to unravel the processes and metabolic constraints induced by the heat, A_{shoot} measurements were refined with concomitant measurements of gas exchange parameters at the leaf level on branches non subjected to the labelling. Particularly, Chlorophyll *a* fluorescence and leaf gas exchange were simultaneously measured on separate middle-level leaves using an open-type portable measurement system (Li-6400, Li-Cor Inc., NE, USA, Fig. 1) equipped with an integrated fluorescence chamber head (Li-6400-40 leaf chamber fluorometer, Li-Cor Inc.) as described in Scartazza et al. (2017). Gas exchange and florescence parameters in the light and in the dark were first registered at ambient (30 °C) temperature in all replicate plants of *A* and *H* group. Then, the leaf temperature was allowed to rise up to 45 °C by means of the integrated Peltier coolers control temperature. Gas exchange and fluorescence parameters were measured approximately 3 h after the temperature was stabilized, corresponding to a middle point of the labelling timing. Instantaneous measurements of steady state leaf CO_2 assimilation rate (A_{leaf}), stomatal conductance (g_s), intercellular CO_2 concentration (C_i), transpiration rate (E), actual photon yield of PSII photochemistry (Φ_{PSII}) and non-photochemical quenching (NPQ) were recorded. Measurements were carried out at CO_2 concentration of 400 μmol mol^{-1} and PPFD of 1000 μmol m^{-2} s^{-1}, reproducing the conditions in the branch chamber subjected to labelling. The value of Φ_{PSII} was determined as $\Phi_{PSII} = (Fm' - F')/Fm'$, where F' is the fluorescence intensity emitted by the leaves under actinic light exposition, whereas Fm' is the maximum fluorescence intensity emitted after superimposing a saturating light flash (8000 μmol m^{-2} s^{-1} for 0.8 s) during actinic illumination. The Stern-Volmer NPQ was calculated as $NPQ = (F_m/F_m') - 1$ (Bilger and Björkman, 1990). The maximum PSII photochemical efficiency (F_v/F_m) and leaf dark respiration (R_{leaf}) were determined on leaves after at least 20 min of dark acclimation (Santaniello et al., 2017). The value of F_v/F_m was determined as $F_v/F_m = (F_m - F_0)/F_m$, where F_m and F_0 represent the maximum ad the minimal fluorescence intensity emitted by the leaves in the dark-adapted state, respectively.

2.2. Preparation of plant material for $\delta^{13}C$ measurement

Water soluble sugars were extracted from roots and leaves of *Eucalyptus* following Brugnoli et al. (1998). Briefly, 100 mg of plant tissue were homogenized in 1.5 mL of distilled water. Subsequently, the homogenates were incubated at 80 °C for 10 min and then centrifuged at 14 000 *g* for 5 min. Supernatants were collected immediately into the new tubes. The procedure was repeated three times. Ion exchange chromatography was used to purify the obtained extracts. Two types of resins, Dowex 1 × 2 Cl (Sigma 44290) and Dowex 50WXB-100 (Sigma 44290) were utilized to eliminate the charged compounds. Prior to the procedure, the resins were washed with distilled water to eliminate any contamination. Purified samples were freeze dried and processed on IRMS Isoprime (Isoprime, Cheadle, UK) coupled to elemental analyzer (NA1500, Carlo Erba, Milan, Italy) for $\delta^{13}C$ determination. 0.1 mg - 0.2 mg of dry material of each sample was used for ^{13}C measurement. Isotope ratios of C ($R = {}^{13}C/{}^{12}C$) were measured in order to calculate $\delta^{13}C$ referring to the VPDB standards as: $\delta^{13}C = R_{sample} / R_{standard} - 1$. The precision of sample measurements was better than 0.1‰.

2.3. Extraction of starch with one step HCl digestion

The residual material after the sugars extraction was washed several times with ethanol (1.0 mL, 80% v/v) at 80 °C for 10 min to remove the pigments. When the pellet became uncolored 1.0 mL of HCl 20% (w/w) was added to solubilize starch (Brugnoli et al., 1988). The samples were shaken for 10 min in ambient temperature and centrifuged at 14 000 *g* for 20 min. The supernatants were collected into 12 mL tubes. The procedure was repeated two times. 7.0 mL of pure methanol was added to 3.0 mL of the obtained extracts. Subsequently, samples were stored at 5 °C overnight to precipitate the starch. After incubation samples were centrifuged at 14,000 *g* for 10 min at 4 °C, washed with distilled water and freeze dried. Desiccated samples of starch were used for ^{13}C isotope detection as it was described for sugar fractions (Scartazza et al., 2013).

2.4. Statistics

The study was performed with three replicates for each treatment. All statistical analyses were done using the software STATISTICA 7 for Windows (StatSoft Inc., Tulsa, OK, USA). The number of replicates was too low for statistical elaboration with parametric tests. The non-parametric Mann-Whitney test was applied to analyze the differences between the treatments in pools and isofluxes alongside the chasing period as well as the differences in the enrichment of plant metabolites and soil between the treatments. The differences between the slopes and the intercepts of the relationships were evaluated through analysis of covariance (ANCOVA).

3. Results

3.1. Gas exchange parameters and isofluxes

A_{shoot} was affected by the heat pulse decreasing gradually during 7 h of treatment and reaching 20% of its pre-heated value by the end of the light and heat period (Fig. 2). This flux did not reach a steady state during the measurements at elevated temperatures. A_{leaf} measured 3 h after the heating start on the separate shoots was positively related to stomatal conductance (g_s, Fig. 3a) and negatively to R_{leaf} measured in the dark (Fig. 3b). Heat pulse induced a reduction in the actual photochemical efficiency of photosystem II (Φ_{PSII}) that was related to an increase in non-radiative energy dissipation capacity, estimated as NPQ (Fig. 3c). Moreover, F_v/F_m of dark-adapted leaves did not show any statistical difference between *A* (0.80 ± 0.01) and *H* (0.78 ± 0.01).

R_{shoot} boosted by almost 200% immediately after the temperature rise (Fig. 2 and 4a). Similarly to A_{shoot}, it did not reach a steady state and was gradually declining while plants were heated. The potential C balance at the shoot level expressed as the ratio of A_{shoot} to R_{shoot} was also constantly declining since the start of the heat pulse, with a sudden drop in the first hour after the treatment starts (Fig. 2). With night onset and temperature decline, R_{shoot} decreased and established at lower rates

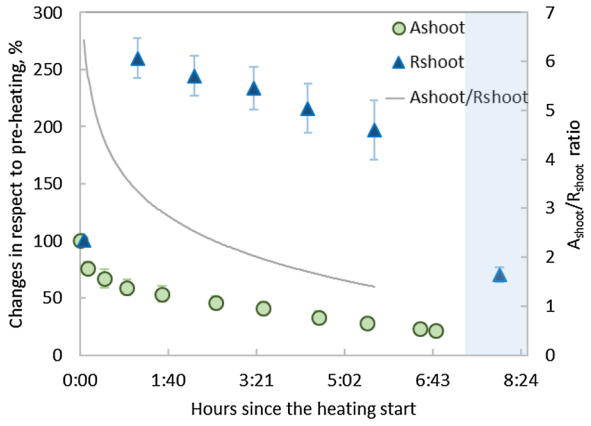

Fig. 2. Dynamics of shoot net CO_2 assimilation (A_{shoot}, mean ± SE) and shoot respiration (R_{shoot}, mean ± SE) in the branch chamber since the onset of the heat pulse expressed in percentage of the pre-heated value. Data are pooled together from plants of ambient (*A*) and heating (*H*) treatments (n = 6). Values for *A* treatment are coming from the second labelling day when the plants were heated. The desired temperatures were reached already from the first reported in the figure value and maintained stable during the whole day. Line is the curve fitted on the variation in the ratio of A_{shoot}-to-R_{shoot}. Grey area refers to the night time (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

than pre-heated values ($p < 0.05$). For *H* plants, during the first night after labelling, R_{shoot} was lower than that of *A* plants ($p < 0.05$), which were just shortly subjected to the heat pulse (Fig. 4a). In the second night, after an equal exposure of both treatments to the heat pulse, R_{shoot} did not differ between *H* and *A* plants.

^{13}C enrichment of R_{shoot} was highly variable among single replicates but the common pattern for all *A* plants was a time of the $\delta^{13}C$ in CO_2 peak, which happened 16 h after the labelling start, in the night hours (Fig. 4b). For *H* plants, $\delta^{13}C$ peaks in R_{shoot} were smoothed and significantly lower compared to *A* plants ($p < 0.05$). Drops in $\delta^{13}C$ rates of R_{shoot} were observed with the light onset and while passing from 30 °C to 45 °C, further maintaining a steady state in *H* treatment and declining slightly in *A* treatment until the next day and light onset. Once the ^{13}C enrichment of respiration is converted to mg notation, exC (Fig. 4b, insert), its pattern follows the R_{shoot} dynamics, boosting contemporary to respiration increase so that the $\delta^{13}C$ of the flux remains relatively stable in both treatments in the moment when the plants pass from ambient to heat pulse temperatures. On the second day after the labelling, at heat pulse onset, R_{shoot} was equal between the treatments but its ^{13}C enrichment was different (Fig. 4a, b) and proportional to net assimilation rate of the branches during the labelling (Fig. 5).

In *A* plants ^{13}C was allocated downwards with the phloem flow and appeared in the trunk respiration flux already 7 h after the labelling with peak values registered 29 h after the labelling (corresponding to translocation velocity in the range of 0.17 - 0.04 m h^{-1}) (Fig. 4c). In *H* plants there were never measured considerable ^{13}C amounts in R_{trunk}: $\delta^{13}C$ was slowly increasing but the maximum differences with pre-labelling rates did not exceed on average 11‰.

R_{trunk} was characterized by a strong diurnal pattern. Minimum rates were measured during the night and the gradual increase was registered generally starting from around 4 a.m. until reaching its maximum during midday hours (Fig. 4d). R_{trunk} was similar in *H* and *A* plants.

3.2. Organs and compound specific analyses

Soluble sugars extracted from labelled new and old leaves were enriched to a similar extent in both treatments after 54 h of the chasing when the labelled shoot was harvested (Fig. 6a). Instead, lower $\delta^{13}C$ was measured in the sugars of *H* plants extracted from the woody branches immediately below the labelled leaves ($p < 0.05$). Whilst

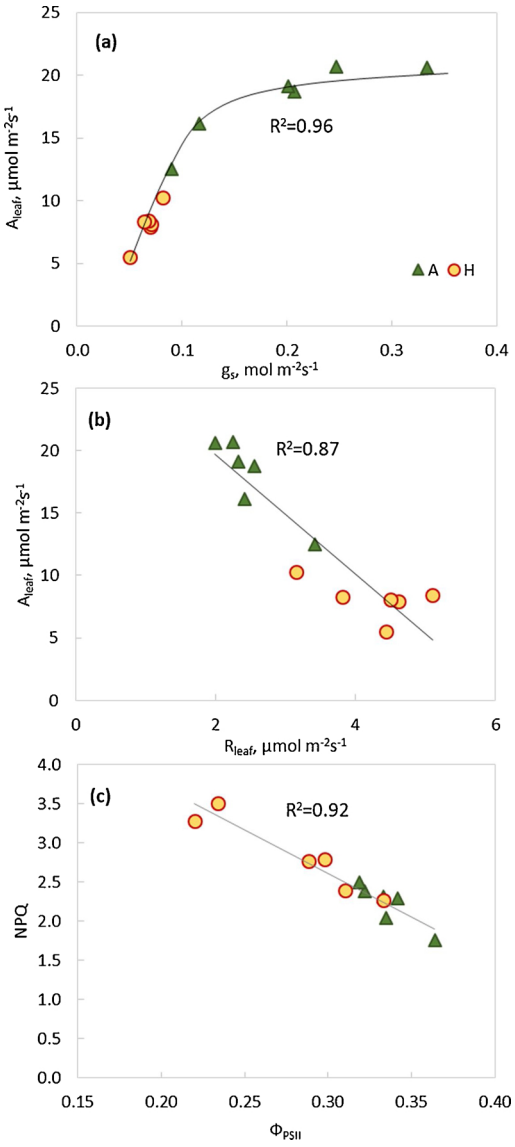

Fig. 3. (a) Leaf net CO_2 assimilation (A_{leaf}) plotted versus stomatal conductance (g_s); (b) A_{leaf} plotted versus leaf respiration in the dark (R_{leaf}); (c) photochemical efficiency of photosystem II (Φ_{PSII}) plotted versus non-radiative energy dissipation capacity estimated as non-photochemical quenching (NPQ). All plants were treated at the same way, measuring all the parameters first at ambient temperature of 30 °C (*A*) and then at 45 °C (*H*) (n = 6). All regression lines are significant at $p < 0.0001$.

reserves enrichment was not visibly affected by the treatment in the new growing leaves, the ^{13}C amount in starch of the old leaves was significantly decreased by the heat pulse (Fig. 6b, $p < 0.05$). Lower starch enrichment was measured also in the woody branches of the labelled shoots ($p < 0.05$).

Enrichment of soluble sugars in leaf and branch tissues 54 h after the labelling was related to A_{shoot} during the labelling (Fig. 7a). The strength of the relationship was higher and the slope was steeper for branch tissues ($p < 0.05$), indicating that proportionally more C is transferred away from leaves characterized by higher photosynthesis rates. Instead, plants with the lowest branch enrichment maintained high investments of ^{13}C to R_{shoot} almost 2 days after the labeling (Fig. 7b).

Label was transported from the point of assimilation also in upward direction and was found in the new leaves of shoots growing above the labelled shoot and in those growing at the same height (Fig. 8a, b). No label was found in non-labelled old leaves in either location within the

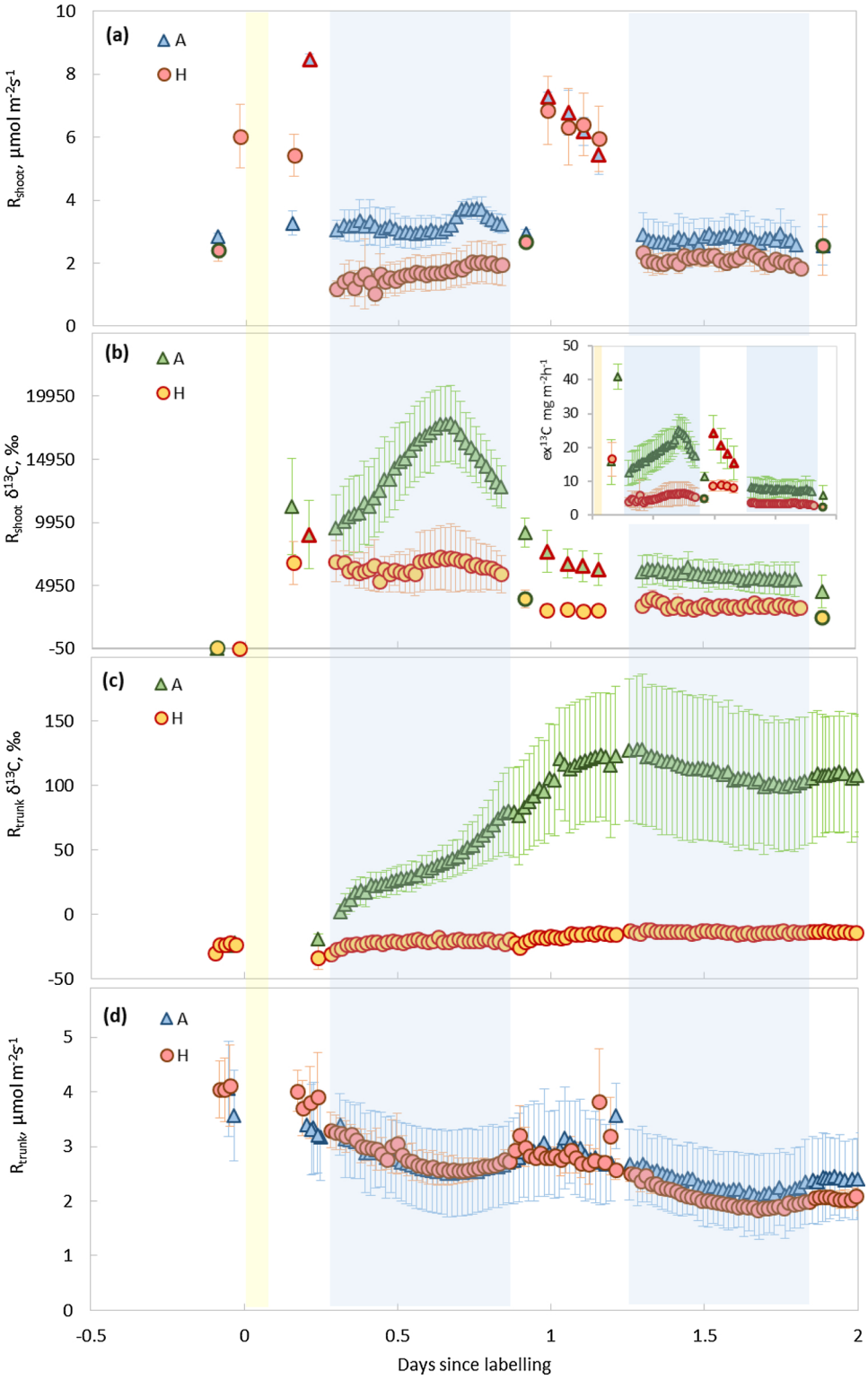

Fig. 4. Shoot respiration ($R_{shoot,}$ mean ± SE) (a) and its C isotope composition (δ^{13}C, mean ± SE) (b); trunk respiration ($R_{trunk,}$ mean ± SE) (d) and its δ^{13}C (mean ± SE) (c) in plants of ambient (*A*) and heating (*H*) treatments (n = 3). Insert – excess of ^{13}C (ex^{13}C, mean ± SE) in R_{shoot}. Labelling time is marked in yellow. Grey areas refer to the night time. Change of the temperature in *A* and *H* treatment is indicated by the change of the border thickness and colour: *H* plants measured at ambient temperature in day time are marked with thick green border; *A* plants measured at elevated temperatures are marked with thick red border (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

canopy (Fig. 8c, d). Transfer of the label upward was observed just in two replicate plants and was independent from heating and assimilation rate of the source leaves during the labelling (Fig. 7a, blue circles). Plants with upward ^{13}C transfer were instead characterized by the highest enrichment of sugars in woody branches immediately beneath the labelling point in respect to labelled leaves. ^{13}C enrichment of roots and R_{mic}, used as a proxy of microbial community enrichment, followed patterns of R_{trunk} enrichment: δ^{13}C peaked in roots and R_{mic} in *A* plants on the second day after the labelling, whereas in *H* plants the label was detected in very minor amounts on the last sampling event (Fig. 8e, f).

4. Discussion

4.1. Heat pulse and gas exchange

While the effects of severe heat shock on gas exchange parameters are widely described in the literature, to our knowledge this is the first time a detailed online allocation assessment of newly assimilated C

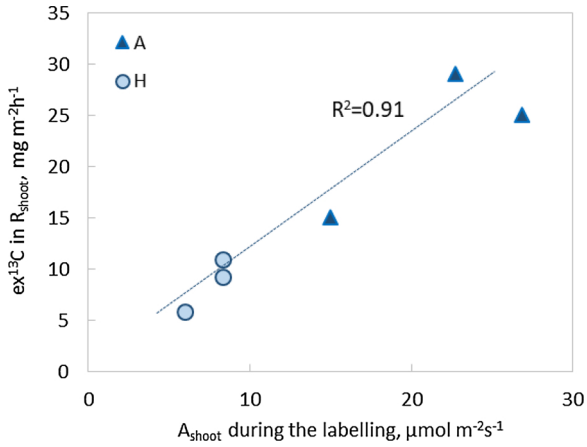

Fig. 5. Excess of ^{13}C ($ex^{13}C$) measured in shoot respiration (R_{shoot}) on the second day after the labelling after the onset of the heat pulse plotted versus shoot net CO_2 assimilation (A_{shoot}) during the labelling in plants of ambient (*A*) and heating (*H*) treatments (n = 3). Regression line is significant at $p < 0.01$.

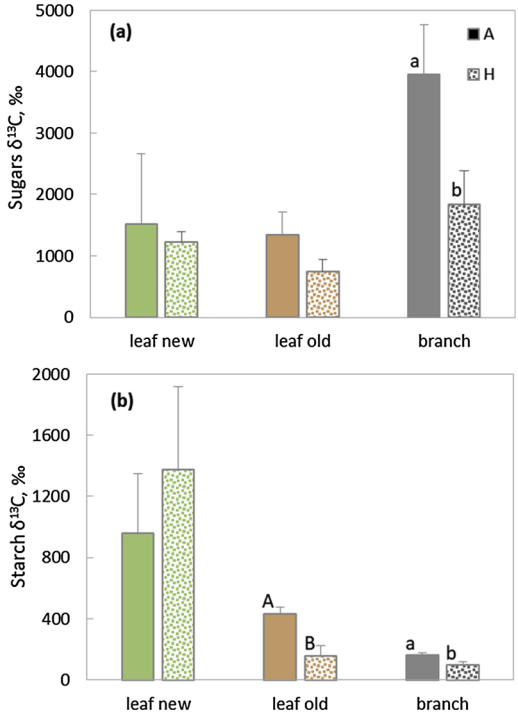

Fig. 6. C isotope composition ($\delta^{13}C$, mean ± SE) of (a) soluble sugars and (b) starch extracted from labelled new leaves, old leaves and woody branches in plants of ambient (*A*) and heating (*H*) treatments (n = 3). Letters above the columns indicate the changes between the treatments at $p < 0.05$.

between compartments in tree-soil-atmosphere continuum is performed in the conditions of the severe heat stress. Temperature optimum for photosynthesis generally corresponds to daytime temperature in the plant natural growing environment and is in the range of 20–30 °C for species growing in various climate zones (Medlyn et al., 2002; Cunningham and Read, 2003; Hozain et al., 2009; Vargas and Cordero, 2013; Yamori et al., 2014). In agreement with these values, *Eucalyptus* assimilation rate declined at 45 °C (Fig. 2). However, similarly to some tropical species, *Eucalyptus* still maintained a positive net assimilation during the whole chasing period (Vargas and Cordero, 2013). The reasons of the decline are multiple and among monitored parameters encompass stomatal closure (Fig. 3a), increased mitochondrial respiration losses (or respiration in the dark Fig. 3b) and reduction of PSII photochemical efficiency (Fig. 3c). Other studies also reported a block

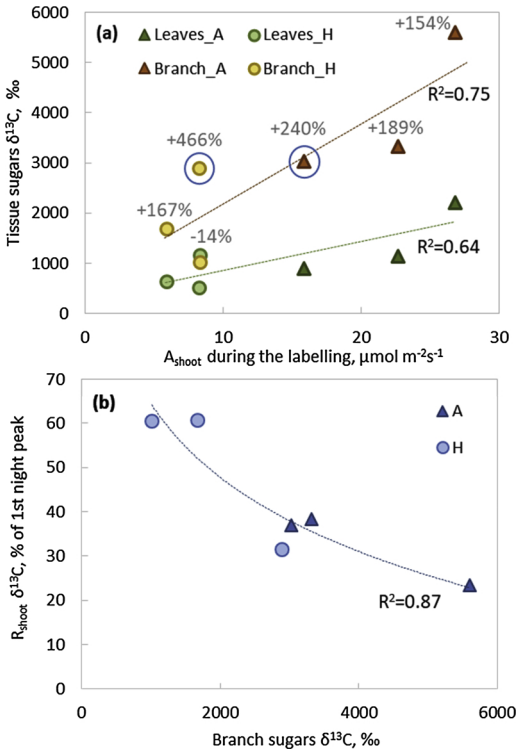

Fig. 7. (a) C isotope composition ($\delta^{13}C$) of soluble sugars extracted from old leaves and woody branches 54 h after the labelling in relation to shoot net CO_2 assimilation (A_{shoot}) during the labelling in plants of ambient (*A*) and heating (*H*) treatments (n = 3). Percentages in grey correspond to percentage increase of branch sugars enrichment in respect to leaf sugars enrichment. Replicates with upward allocation of label are marked with blue circles; (b) C isotope composition ($\delta^{13}C$) of shoot respiration (R_{shoot}) during the second night after the labelling (maximum value) expressed as % of the first night peak in $\delta^{13}C$ of R_{shoot}, plotted in relation to woody branch enrichment 54 h after the labelling (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

of the *Rubisco* activase enzyme and increase in photorespiration (Haldimann and Feller, 2004; Hozain et al., 2009; Jin et al., 2010). It is important to note as the *Eucalyptus* leaves exposed to high temperature were able to increase the dissipation of the excess energy at PSII as heat, as suggested by the relationship between Φ_{PSII} and NPQ (Fig. 3c). This photoprotective mechanism allowed to counteract the excessive excitation energy avoiding irreversible damages at the photosynthetic apparatus, as suggested by the F_v/F_m values of leaves subjected to heat pulse, not statistically different from those of non-stressed leaves. In contrast, we did not find signs of the leaf latent cooling, measured as increase in stomatal conductance and transpiration and decline in leaf temperature during severe heat stress for species like *Eucalyptus parramattensis, Populus deltoids* and *Pinus taeda* (Urban et al., 2017; Drake et al., 2018). We hypothesize that *Eucalyptus camaldulensis* do not rely on this particular mechanism to tolerate heat or, more probable, that the activation temperature for latent cooling was not achieved at 45 °C.

R_{shoot} and R_{leaf}, boosted by 200% with high temperature, confirming different temperature optima for CO_2 assimilation and respiration (hypothesis 1, Fig. 2 and 3b; Teskey et al., 2015). Although respiration maximum for *Eucalyptus* spp. was reported at temperatures above 50 °C (O'Sullivan et al., 2013), the established elevated respiration rates were not maintained by the plants, highlighting possible acclimation of respiration either substrate limitations. Hüve et al. (2012) observed a decline in the amount of leaf starch already after 30 min of the darkening for *Populus* exposed to 50 °C which supports the substrate limitation hypothesis. The C balance of the branch, expressed as A_{shoot}-to-R_{shoot} ratio was constantly declining, suggesting C losses from the

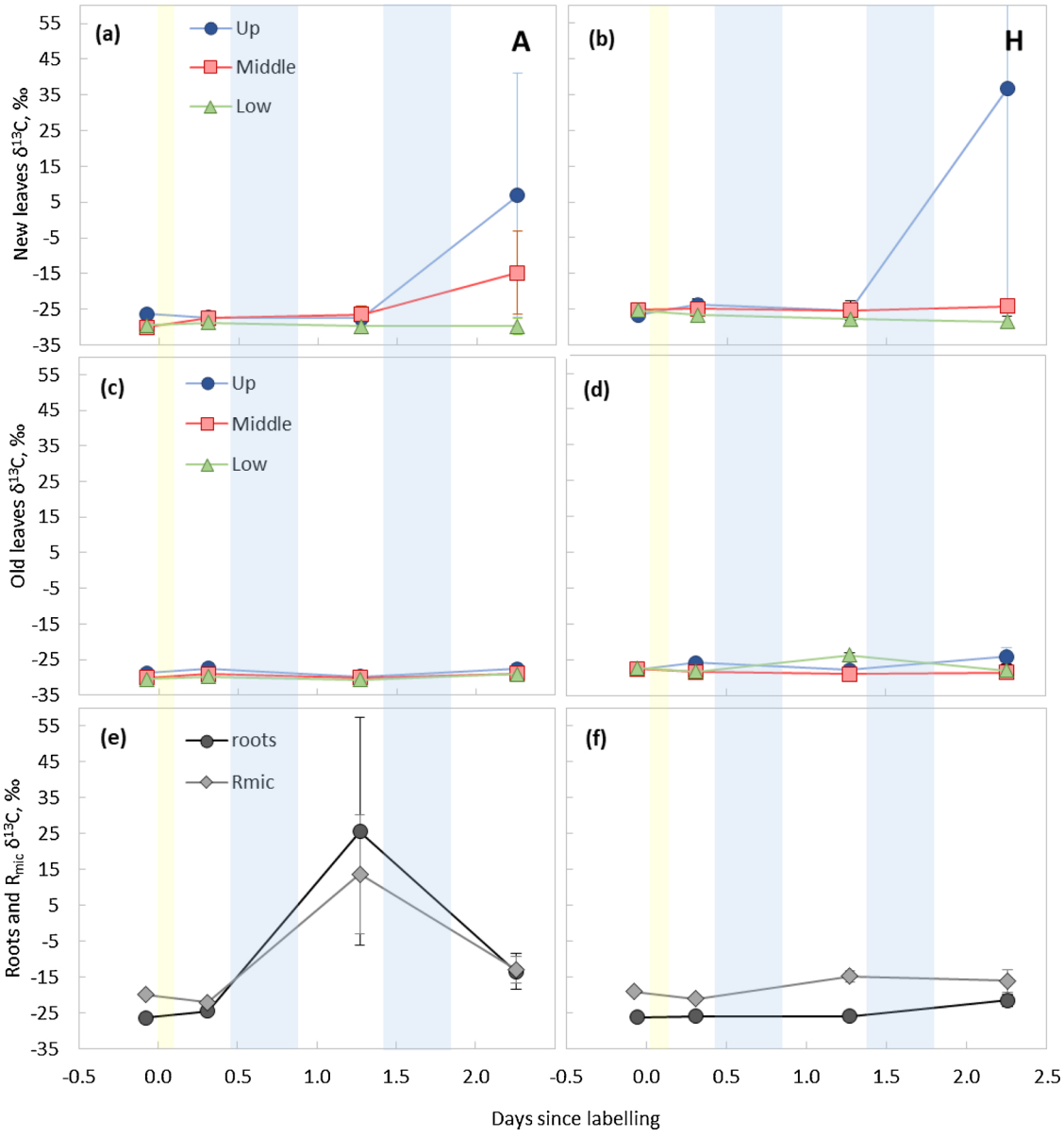

Fig. 8. C isotope composition ($\delta^{13}C$, mean ± SE) of soluble sugars extracted from non labelled new leaves (a–b) and old leaves (c–d) collected from three plant heights (Up – upper canopy, Middle – middle canopy, Low – low canopy height); and that of roots and microbial respiration R_{mic} (e–f) in ambient (*A*: a, c, e) and heating (*H*: b, d, f) treatments (n = 3). Labelling time is marked in yellow. Grey areas refer to the night time (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

system with the increase of heat period.

4.2. Leaf and branch-level allocation of ^{13}C

The presence of multiple C pools with various turnover rates was highlighted in some tracer studies on non-stressed woody vegetation (for review Epron et al., 2012; Hartmann and Trumbore, 2016). Generally, contribution of two-to-three pools to respiration fluxes of different origin is highlighted: the contribution of the fast cycling pool is high in the first days (Plain et al., 2009; Epron et al., 2012), a medium cycling pool (or transient storage) with mean residence time from weeks to a season (Plain et al., 2009; Carbone and Trumbore, 2007; Carbone et al., 2013) and a long-cycling pool (or storage) with mean residence time of one year or more (Vargas et al., 2009; Carbone et al., 2013; Muhr et al., 2016). The long-lasting storage pool has a buffering function and its contribution to plant metabolic activity at normal conditions of growth should be small (Gessler and Treydte, 2016). Recent experiments suggest that stored C can have a "dominant" role when the metabolic interactions within the plant are impaired by stress or during particular phases of the growing season like spring re-sprouting (Carbone et al., 2013; Zang et al., 2014; Hasibeder et al., 2015; Muhr et al., 2016; Von Rein et al., 2016).

The shape of R_{shoot} enrichment curves evidence an involvement of various fast cycling labelled substrates in diurnal respiration course and their variable contribution between *H* and *A* treatment (Fig. 4b). Generally, among these substrates are included soluble carbohydrates and fast cycling transient starch (Gessler et al., 2008; Barthel et al., 2011; Gavrichkova et al., 2011). Enrichment of R_{shoot} in *A* treatment was characterized by a bell-shape diurnal pattern. The highest peak was reached by night, 16 h after the labelling, and is in line with the release of the labelled transitory starch formed during the day in the chloroplasts and broken down during night (Gessler et al., 2008; Barthel et al., 2011; Gavrichkova et al., 2011). The form of the peak suggests that this transitory starch pool is not well mixed but could be rather

characterized by an "onion" structure. "Peels" of starch are accumulating gradually during the day and are gradually consumed during the night (Smith et al., 2005), so that decline in R_{shoot} enrichment occurs when starch formed during the labelling procedure is "peeled off" and is further substituted with not enriched starch formed prior to the labelling. The same pattern was documented for leaves in poplar saplings (Ghirardo et al., 2011) and at root level in beech (Barthel et al., 2011). The relative stability of $\delta^{13}C$ in R_{shoot} in *H* plants suggests that C is provided from a well-mixed pool of C in contrast to *A* treatment. In accordance with hypothesis 2, synthesis of the transient starch and long-term storage compounds seems to be compromised under heat stress, as suggested also by leaf and branch starch enrichment (Fig. 6b) and diurnal R_{shoot} enrichment curve. To compensate, relative ^{13}C allocation to leaf soluble sugars was maintained at the level of *A* plants in expense also to branch allocation (Fig. 6a). Soluble carbohydrates are precursors of secondary compounds involved in thermotolerance mechanisms such as isoprenoids, lignin, flavonoids, membrane lipids produced in the conditions of increasing temperatures (Kaplan et al., 2004; Velikova and Loreto, 2005; Hartmann and Trumbore, 2016). Soluble carbohydrates dissolved in the cell's cytosol should be also well-mixed (Voet et al., 2006). Given that and in agreement with the diurnal stability of the isotope composition of R_{shoot}, we propose that soluble sugars are the primary components of synthesis and transient-accumulation in the conditions of heat stress.

On the second day after the labelling, *A* and *H* plants were heated contemporary and R_{shoot} established at similar rates in both treatments (Fig. 4a). The contribution of ^{13} C to elevated R_{shoot} was however different between *A* and *H* (Fig. 4b, insert). Although *H* plants tended to keep assimilates within the labelled shoot, a strong relation of $ex^{13}C$ with assimilation rate during the labelling suggests that plants mobilize only a certain part of C from a fast cycling pool in order to support high respiration expenses (Fig. 5). It points on possible involvement of additional C reserves in the conditions of low photosynthetic income and with increase of the duration of the heat stress. The displacement of C and nutrients from storage organs and between the leaves to meet the immediate local demand was confirmed for many woody species (Milla et al., 2007; Palacio et al., 2007; Ghirardo et al., 2011; Gavrichkova et al., 2017, 2018). As discussed above, a progressive restriction of the substrate with temperature may itself limit the respiration which is in agreement with progressive respiration decline after the heat pulse onset (Fig. 1). Hence, our data evidence that, congruent to the hypothesis 2, the contribution of older non-labelled C to R_{shoot} is possible and would depend on the assimilation rate and on the duration of heat.

4.3. Allocation of ^{13}C in the plant-soil system

Heat pulse compromised not only the synthesis of the storage compounds but also the ^{13}C allocation to heterotrophic organs (hypothesis 3). The ^{13}C appearance in woody branches beneath the labelled leaves was not related straightly to the heating itself but rather to leaf assimilation rate, meaning that this process is likely source driven (leaves with higher photosynthesis deliver a higher portion of newly assimilated C to woody branches) (Fig. 7a). In two replicates (one – from *A* and one – from *H* treatment, Fig. 7a blue circles) we observed upward transfer of ^{13}C to new leaves of apical shoots. A_{shoot} in these plants was not particularly high during the labelling, but the assimilates transfer rate to beneath branches was among the highest (Fig. 7a, in grey). Hence, sink attraction force contributes alongside the photosynthesis to assimilates re-allocation from leaves and might be crucial in species with indeterminate growth such as *Eucalyptus* to create new tissues. Growing leaves and needles together with developing fruits are strong C sinks and require C acquired by fully developed leaves (Schneider and Schmitz, 1989; Hoch and Keel, 2006). Transfer of C in upward direction was documented for larch (Schneider and Schmitz, 1989), poplar saplings (Ghirardo et al., 2011), and Mediterranean shrubs (Gavrichkova et al., 2018) by similar pulse labelling approach. To our knowledge, it is the first confirmation for *Eucalyptus*. Given that, apical shoots were not exposed to elevated temperatures, their growth and C sink force were not altered. Under plants natural growing conditions, when the whole crown is exposed to the heat wave, the sink capacity of newly forming tissues will be likely affected too.

Although the label entered the transporting system of *H* plants, its downwards transfer almost ceased, as demonstrated by very low enrichment of R_{trunk} (Fig. 4c), roots and R_{mic} (Fig. 8e, d). The experimental layout excludes any modifications in the phloem conductibility which varies with temperature (Plain et al., 2009) and water supply (Ruehr et al., 2009; Steppe et al., 2015), because the trunks and the major part of the canopies were not exposed to the heat pulse. Consequently, R_{trunk} absolute rates were not affected directly by the heat. Hence, patterns of R_{trunk} enrichment should be related exclusively to the changes in plant allocation strategies within the labelled and heated shoots (hypothesis 3). We could expect that in the conditions of the whole-tree stress, the downward C transfer will be blocked which will induce substantial impact on the internal C dynamics of heterotrophic organs.

R_{trunk} was characterized by a strong diurnal periodicity in both treatments with the highest rates measured during the mid-day hours and the lowest during the night (Fig. 4d). The main drivers influencing R_{trunk} diurnal course are: atmospheric temperature (Yang et al., 2014), substrate amount (Martin et al., 1994), cell growth and CO_2 transport with the xylem sap (Steppe et al., 2015). The last two parameters may bring to a mid-day depression of the respiration flux which was not observed in our experiment. R_{trunk} was not in phase with ambient temperature changes: night time flux starts to increase 2 h earlier than ambient temperature starts to grow (data not shown). Although the delivery of the labelled substrates was also not in phase and occurred later in respect to day time respiration peak (Fig. 4c and d), we still propose it as a primary driver of R_{trunk}. Plants were labelled few hours after switching on the light therefore non-labelled assimilates of that day were delivered to the trunk earlier as compared to the labelled one, so that the peaks in R_{trunk} and in its $\delta^{13}C$ resulted slightly shifted in time. Taken into account the velocity of the phloem transport, the second day increase in the $\delta^{13}C$ of R_{trunk} should correspond to the detection of the label released after a night time break down of starch.

Block of belowground C transfer explains also the results of the experiments which addressed growth issues in plants subjected to repeated heat waves, where growth and root biomass were significantly reduced in *Quercus rubra* and *Pinus taeda* (Bauweraerts et al., 2014). While in early summer belowground C allocation is generally lower in respect to other periods of plant phenological development, it increases in late summer as demonstrated on poplar, larch and beech before the leaf fall (Horwath et al., 1994; Kagawa et al., 2006; Scartazza et al., 2013, 2015). Hence, the period of the exposure of a plant to a heat wave will determine the consequences and the severity of the impact on belowground allocation and on the whole plant C budget. Given that, already an immediate short-term impact after few hours of the heat pulse, common e.g. during the daytime in summer in Mediterranean, is substantial.

5. Conclusions

In conclusion, heat pulse simulating heat waves occurring in the Mediterranean area strongly affects C gain and allocation in plant-soil-atmosphere continuum. To the detriment of reserves, plants maintain elevated synthesis level of soluble carbohydrates in order to cover increased shoot respiration costs and to possibly enhance thermoprotection mechanisms. Assimilates are mainly retained in leaves and allocation to other organs is possible only in case of their strong sink capacity, which however can be hardly maintained under stress conditions. Carbon limitation of heterotrophic organs induced by heat mediated missing C displacement from leaves should be covered by local C reserves (e.g. from starch). We expect that prolonged heat pulses

during the end of the growing season, characterized for many species by reserves accumulation, may shift the process to reserves depletion. Consequently, strong heat waves common in the last years in Mediterranean and expected in other climates worldwide, have not only the direct and immediate effects on plant C gain and internal C allocation but may affect the re-sprouting and plant survival in the next year because of the consumption of storage compounds.

CRediT authorship contribution statement

O. Gavrichkova: Conceptualization, Data curation, Formal analysis, Investigation, Funding acquisition, Methodology, Validation, Writing - original draft. **A. Scartazza:** Conceptualization, Formal analysis, Investigation, Data curation, Validation, Writing - original draft. **G. Guidolotti:** Formal analysis, Funding acquisition, Methodology, Validation, Writing - review & editing. **Y. Kuzyakov:** Conceptualization, Funding acquisition, Methodology, Writing - review & editing. **L. Leonardi:** Data curation, Methodology. **M. Mattioni:** Software, Data curation, Methodology, Writing - review & editing. **J. Nawrocka:** Methodology, Formal analysis, Data curation, Writing - review & editing. **E. Pallozzi:** Conceptualization, Data curation, Formal analysis, Methodology, Software, Writing - review & editing. **M. Skwarek:** Methodology, Formal analysis, Data curation, Writing - review & editing. **M. Tomczyńska:** Methodology, Formal analysis, Data curation, Writing - review & editing. **C. Calfapietra:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.envexpbot.2019.02.019.

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